Application of Probes in Live Cell Imaging

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Abstract: Live cell imaging (LCI) is the approach of non-invasively analyzing dynamic processes in living cells using state-of-the-art microscopy and computer vision techniques. LCI provides exciting and novel insights into cell biology. The present review summarized LCI research in recent years and detailed the role of probes in LCI. We discussed the basic principle and development in LCI technology, including fluorecence resonance energy transfer, fluorescence lifetime imaging microscopy, atomic force microscopy, scanning ion conductance microscopy, confocal microscopy, two-photon excitation fluorescence microscopy and Cell-IQ® System . Then we listed several probes such as green fluorescent protein and quantum dots which are the most widely used probes. Proteins, organelles, nucleotides and other chemicals related to cell biology may all be potential targets. We also discussed the advantages and disadvantages of different probes. Although specific probes in LCI still need to be explored, the application of LCI may be attractive for better understanding of cellular functions.

Keywords: Live cell imaging, probes.

INTRODUCTION

One of the main challenges of modern biochemistry and cell biology is to observe molecular dynamics in the functional context in live cells or in situ and track ongoing molecular events with maximal spatial and temporal resolution within subcellular compartments in order ro minimize the interference with tissue biology, a future development for in situ imaging [1]. Live cell imaging (LCI) is the approach of non-invasively analyzing dynamic processes in living cells using state-of-the-art microscopy and computer vision techniques [2]. LCI research provides exciting and novel insights into cell biology. Observing a biological event as it unfolds in the living cell offers an unique insight into the nature of the phenomenon under study [3]. In LCI, the probes of microscopy are the key part. Up to now, various molecules and occuring processes in live cells can be detected by different kinds of probes. In this review, we summarized recent development in LCI and discussed the role of probes in LCI.

THE BASIC PRINCIPLE OF LCI

The underlying molecular interactions in a coordinated manner are orchestrated with an extensive degree of spatiotemporal regulation. Despite the extensive and highly complex networking involved, the fundamental physicochemical basis corresponds to a limited set of reactions: binding, conformational transition, covalent modification, and transportation. LCI can provide information about all of these molecular processes with high specificity and sensitivity *via* appropriate probes and instruments (See Fig. **1**).

Fluorescence labeling permits the study of the complex and delicates relationships existing between structure and function in biological systems [4, 5]. Optical microscopy has evolved to three dimensional and four-dimensional image formation and analysis tool and offered a means to probe various processes at submicron and nanometer levels [6, 7]. Here we mainly introduced the techniques and applications frequently used in human LCI.

Fluorecence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (FRET), or more exactly Förster (the name of Theodor Förster, the discoverer of the phenomenon) [8] resonance energy transfer is applied extensively in research science and technology, generally but not exclusively as a "nanoruler" with a dynamic range of 0-10nm. As a photophysical phenomenon, FRET is generally regarded as a competitor with other forms of radiative and non radiative decay from the first excited singlet state of a fluorophore [9]. This technique is globally used for studying protein interactions within live cells. However, its effectiveness and sensitivity can be reduced by photobleaching, cross talk, autofluorescence, and unlabeled, endogenous proteins. *In vivo* imaging studies on the green fluorescent protein-alkylguaninetransferase (GFP-AGT) (NitroBIPS) complex, employing optical lock-in detection of FRET, allowed high resolution of FRET efficiencies below 1%, equivalent to a few percent of donor-tagged proteins in complexes with acceptor-tagged proteins [10].

Fluorescence Lifetime Imaging Microscopy (FLIM)

FRET provides the motivation for a large fraction of fluorescence lifetime imaging microscopy (FLIM) experiments. Fluorescence imaging of green fluorescent protein (GFP) may be utilized to locate proteins in live cells, then FLIM can probe the local microenvironment of proteins. FLIM to GFP-tagged proteins are applied at the cell surface and at an inhibitory natural killer (NK) cell immunological synapse for detecting small but significant differences in

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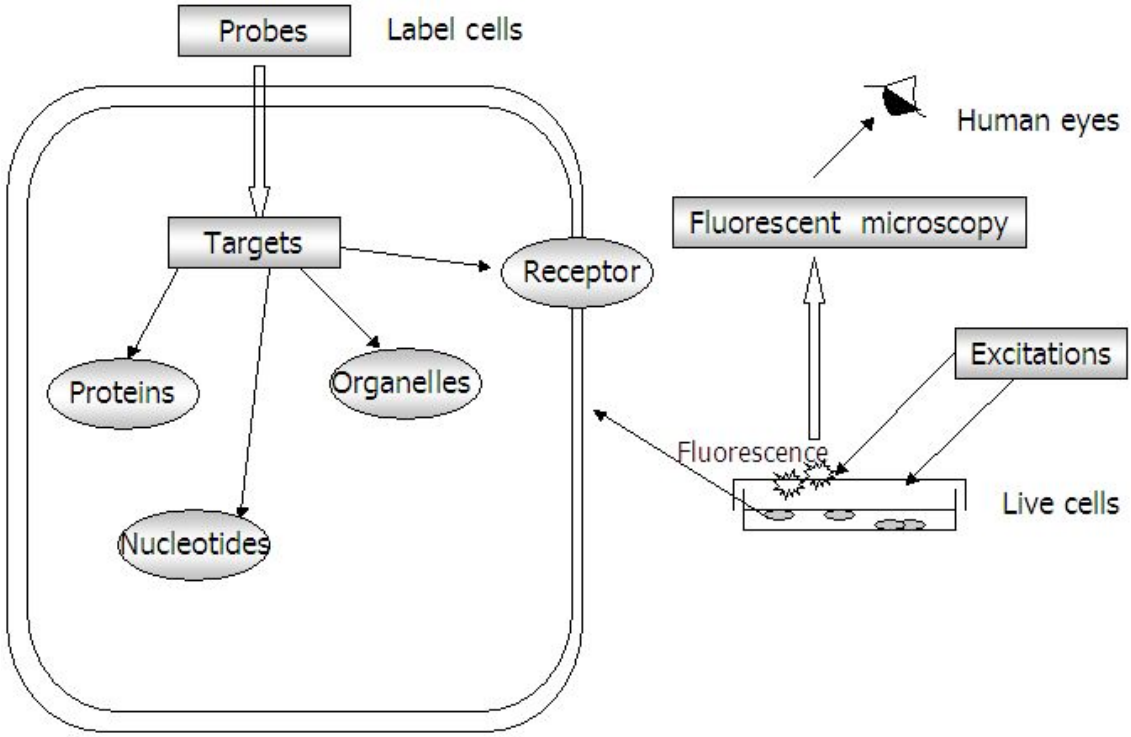

Fig. (1). Overview of the general principle for live cell imaing. Probes are constructed to label specific targets, i.e proteins, receptors, nucleotides etc. Upon stimulations of the cells with potential endogenous and exogenous chemicals, the increase or decrease signals, such as fluorescence are observed by microscopes.

fluorescence lifetimes, suggesting that FLIM could be broadly useful in imaging discrete membrane environment for a given protein [11].

Atomic Force Microscopy (AFM)

The atomic force microscope (AFM) is a powerful tool to investigate surface and submembranous structures of living cells under physiological conditions at high resolution. This property enabled us to study the interaction between live hepatic NK cells, also called pit cells, and colon carcinoma cells *in vitro*. AFM can be utilized to probe early cytotoxic effects of effector cell to target cell contact in nearby physiological conditions. Other routine cytotoxicity tests were also used to detect the first cytotoxic effects after 1.5-3 h co-incubation at the earliest [12].

As AFM imaging of live specimens become more commonplace, at least two important questions are raised: whether live specimens remain viable during and after AFM and whether there is a transfer of membrane components from the cell to the AFM probe during probe-membrane interaction. Although AFM imaging of live cells in culture didn't affect long-term cell viability, there were substantial probe-membrane interactions that lead to transfer of membrane components to the probe [13]. During LCI *via* AFM, the interactions between the AFM probe and the membrane lead to distorted cell images. An image correction method was developed based on the force-distance curve [14] and the modified Hertzian model [15]. Results have shown that the model could be used to recover up to 30% of the actual cell height depending on the loading force. The accuracy of the model was also investigated with respect to the loading force and mechanical property of the cell membrane [16].

Scanning Ion Conductance Microscopy

Scanning ion conductance microscopy (SICM) provides unique information about living cells, which help us to understand more cellular functions. It is a potentially powerful tool for research on living renal cells [17] and has been used to elucidate how a functional epithelial monolayer maintains its integrity [18]. Hopping mode SICM allowed noncontact imaging of the complex three-dimensional surfaces of live cells with resolution better than 20 nm. Thus, this technique could exam nanoscale phenomena on the surface of live cells under physiological conditions [19].

Confocal Microscopy

The application fluorescence to confocal and tow-photon excitation (2PE) optical microscopy has led to terrific advances in the study of biological systems from the three-dimentional (3D) micro-spectroscopic level down to single molecule detection (SMD) schemes. The advent of the confocal fluorescence microscope substantially increased comprehension of the structure and functional organizational motifs of biological systems [20]. Shevchuk *et al.* [21] firstly introduced a high resolution surface scanning confocal microscopy technique that enables imaging of endocytic pits in apical membranes of live cells, which improved topographical resolution of the microscope together with simultaneous fluorescence confocal detection produces pairs of images of cell surfaces sufficient to identify single endocytic pits.

Two-Photon Excitation (2PE) Fluorescence Microscopy

Two-photon fluorescence microscopy [22, 23] is probably the most relevant advancement in fluorescence optical microscopy since the introduction of confocal imaging [24,

25]. Many commercially available fluorescent probes have been used in multiphoton-based imaging studies despite exhibiting relatively low two-photon absorption cross-section values in the tunability range of ultrafast Ti:sapphire lasers commonly used in multiphoton microscopy imaging. Such properties make these fluorenyl derivatives compelling candidates for multiphoton bioimaging applications, and in particular, as probes or tags for direct covalent linkage onto biomolecules [26].

Cell-IQ® System

Cell IQ system (Chip-Man Technologies Ltd., Tampere, Finland) is a fully integrated continuous LCI and analysis platform, ideal for cell lines, primary cells, co-cultures and mono-player tissue models [27] (see Fig. **2**). Through the proprietary integrated informatics and optics identifies, Cell-IQ visualized and recorded all changes in morphology and physiology down to the single cell during an incubation in real time [28]. Cell-IQ combined long term cell incubation conditions with phase contrast imaging and Machine Vision Technology [29] to automatically identify, analyze and quantify cell morphological features, which allowed researchers to follow the entire culture through all growth phases [27]. This new automated system enabled rapid and reliable analysis of undifferentiated growth dynamics of human embryonic stem cells [30].

PROBE SORTING

Probes are the core part of microscopy. Generally, probes are molecules which can interact with specific targets (proteins, genes, etc.) and can be detected by certain instrument. The advent of genetically encoded markers, such as GFP (see Fig. **3a**) and its variants, has truly revolutionized the biochemical methods required to selectively label molecules or cellular compartments and record their properties in living systems [31, 32]. A multitude of fluorescent indicators and sensors can now be inserted in the genome of various organisms. Advanced chemical methods have also been developed to conjugate biomolecules (proteins, nucleic acids, sugars, etc.) to exogenous probes used as contrast agents [33].

Advanced inorganic nano-materials added new elements to the toolbox with which biological systems can be investigated [34]. Semiconductor nanocrystals, or quantum dots (QDs), are exciting new fluorescent probes in imaging at the single molecule to the whole animal level. They are composed of different semiconductor materials, including the most frequently used one being made of a CdSe core capped by a ZnS shell (Fig. **3b**) [35]. These nanocrystals are capped with surface ligands (bifunctional molecules, amphiphilic polymers, phospholipids, etc.) that render them hydrophilic and provide them with functional properties. These coatings alter their hydrodynamic radii and surface charge, which can drastically affect properties such as diffusion within the cell cytoplasm. The effects of surface ligands on the hydrodynamic radius and on the nanoparticle mobility are complex and strongly dependent on a combination of the inorganic core size, nature and lateral extension of the hydrophilic surface coating. These features are critical for the design of QD-based biosensing assays as well as QD bioconjugate diffusion in live cells [36]. Different probes are for different purposes. We classified several commonly used probes in LCI studies in Table **1**.

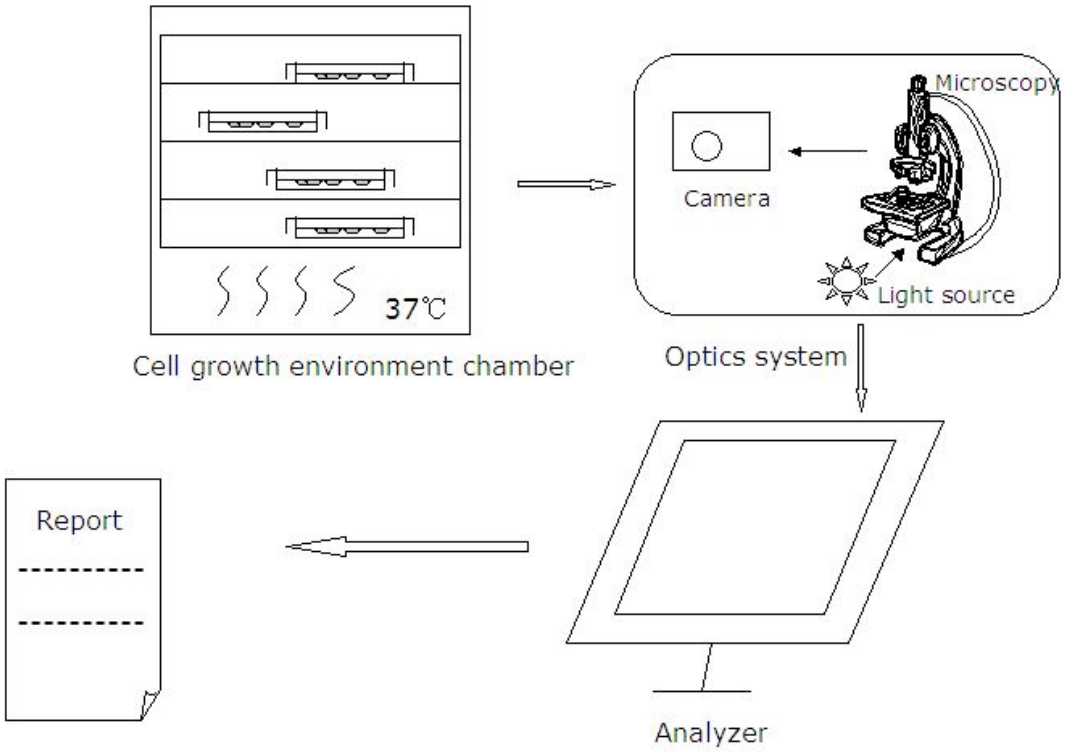

Fig. (2). A scheme drawing of the Cell-IQ® system. This system contains three components. The cell growth environment chamber, where microplates or culture flasks can be maintained under optimal growth conditions of gas, humidity and temperature within Cell-IQ for days or even months. The optics system comprises of a microscope, light source and camera for recording time lapse digital images. The Cell-IQ analyzer software utilizes machine vision to automatically identify and quantify cell types.

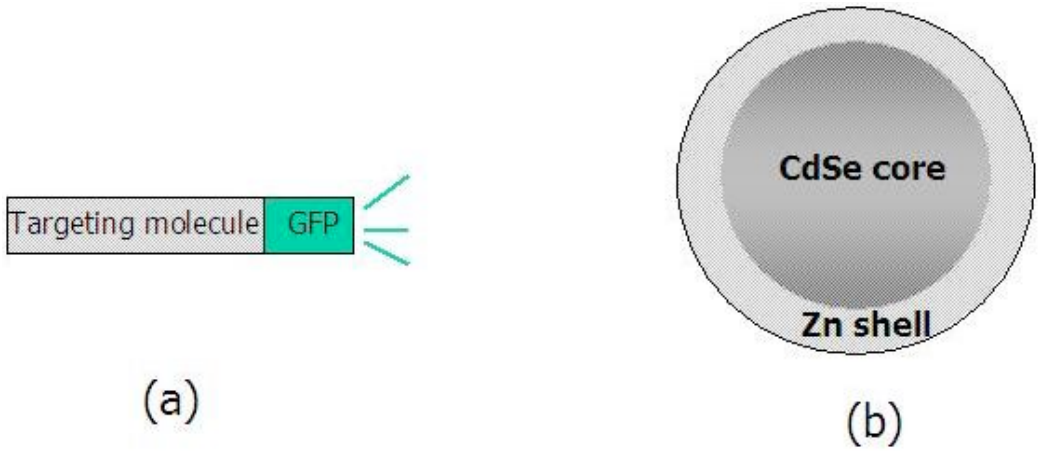

Fig. (3). The structures of green fluorescent protein (GFP) and quantum dot(QDs). (**a**). a scheme of a recombinant protein with a targeting molecule fused with GFP to trace the position of the targeting molecule. (**b**) QDs are inorganic fluorophores and consist of a cadium selenide (CdSe) core with several layers of a thick zinc sulfide (ZnS) shell to improve quantum yield and photostability.

Table 1. The Classification of Probes Commonly Used in Live Cell Imaging

Classification		Reference number
Fluorescent protein	Green fluorescent protein(GFP)	[37-40, 44, 45, 94]
	Near-infrared fluorescence(NIRF)probe	[49]
	Two-photon fluorescent probe	[64, 76]
Nanoparticles	Quantum dot (QDs) probes	[42, 43]
	pH sensitive probe	[51, 52, 95]
	Gold nanoparticles	[83]
	Magnetic nanocrystal	[84]

Proteins

Direct observation of molecular dynamics can provide important information about cellular events that cannot be obtained by other methods. Thus imaging of protein dynamics in living cells becomes an important tool for cell biology to study molecular and cellular functions.

Protease

Determination of the specific roles of proteases in cell migration and invasion requires high-resolution imaging of the sites of protease activity during live cell migration through extracellular matrices. A novel fluorescent biosensor was designed to detect localized extracellular sites of protease activity and to test requirements for matrix metalloprotease (MMP) function as cells migrate and invade three-dimensional collagen matrices. The new type of high-resolution probe provided site-specific reporting of protease activity and insights into mechanisms by which cells migrate through extracellular matrices; it also helps to clarify discrepancies between previous studies regarding the contributions of proteases to metastasis [37]. Protease activity is tightly regulated in both normal and disease conditions. However, it is often difficult to monitor the dynamic nature of this regulation in the context of a live cell or whole organism. A series of quenched activity-based probes which become fluorescent upon activity-dependent covalent modification of a protease target, were used to monitor real-time protease activity in live human cells with fluorescence microscopy techniques as well as standard biochemical methods [38].

Receptors and Ligands on Membrane

An emerging theme in cell biology is that cell surface receptors need to be considered as part of supramolecular complexes of proteins and lipids facilitating specific receptor conformations and distinct distributions, e.g., at the immunological synapse. Thus, a new goal is to develop bioimaging which not only locate proteins in live cells but can also probe their environment. Suhling *et al*. [39] showed that the fluorescence decay of GFP depended on the local refractive index by time-correlated single-photon counting. This novel approach paves the way for imaging the biophysical environment of specific GFP-tagged proteins in live cells. A pulse labeling of AGT-ER(the human estrogen receptor alpha with human O6-alkylguanine-DNA alkyltransferase) fusion proteins with different fluorophores was presented as a novel tool for investigating the functional regulation of nuclear receptors in individual cells [40].

A 99mTc-labeled stromal cell-derived factor-1 (SDF-1) alpha radiotracer could be used as a sensitive and specific

probe for CXCR4 expression *in vivo* and to quantify changes in CXCR4 expression under different physiologic and pathologic states [41]. QDs was used as a probe to detect membrane molecules of interest and was applicable to various cultured cells [42]. Ligand nerve growth factor-bound quantum dots (NGF-QDs) were powerful intracellular probes providing biologists with new capabilities and fresh insight for studying endocytic receptor signaling events in real time, and at the resolution of single or small numbers of receptors in live cells [43].

Intracellular Transport System

High-resolution imaging of cell-based fluororeporters is used to establish and to correlate quantifiable metrics of cell functional endpoints (e.g., cell growth, cell adhesion, cell attachment strength), as well as of intracellular cytoskeletal features (e.g., descriptors of actin organization) on a set of model biomaterial substrates synthesized by combinatorial variations. This quantitative approach of live fluororeporter cell imaging could be valuable for metrology of cell-material interactions [44]. Cytoplasmic dynein 1 is a multi-subunit motor protein responsible for microtubule minus end-directed transport in axons. By constructed GFP-tagged intermediate chains, LCI and comparative immunocytochemical analyses showed that dynein was enriched in the actin rich region of growth cones [45]. Likewisely, a novel fluorescence loss in photobleaching (FLIP)-based approach utilized a synaptophysin-enhanced green fluorescent protein (EGFP) probe that allowed the differential evaluation of the ante- and retrograde transport parameters. The application of an ECFP/VenusYFP fusion FRET couple improved the precision of axonal transport measurements since it combined FLIP and fluorescence localization after photobleaching (FLAP) techniques and eliminated the need for prebleaching images [46].

Proteins in Programmed Cell Death

Programmed cell death, or apoptosis, is an essential event in animal development which is required for normal cellular homeostasis, and the deregulation of apoptotic process is implicated in various diseases. Using small molecules as molecular probes, mechanisms specifically regulating the death of mammalian cells can be investigated [47]. A "smart" activated peptide-based probe was developed and applied for detection of Ras-related farnesyl protein transferase (FPT). Upon farnesylation by FPT, the probe was brought close to a hydrophobic milieu and as a consequence emitted fluorescent light that could be detected by several media, such as fluorescence microscopy, a plate reader, and an optical imaging system [48].

The second generation probe of a cell-penetrating near-infrared fluorescence (NIRF) probe based on an activating strategy was used to detect apoptosis-associated caspase activity *in vivo*. KcapQ, with a modified cell-penetrating peptide sequence (KKKRKV), represented an improved effector caspase-activatable NIRF probe for enhanced noninvasive analysis of apoptosis in whole cells and live animals [49]. Osteoclasts degraded bone matrix by demineralization followed by degradation of type I collagen through secretion of the cysteine protease, cathepsin K. By NIRF probe activated by cathepsin K, Kozloff *et al*. [50] demonstrated noninvasive visualization of bone degrading enzymes in models of accelerated bone loss, which might provide a means for early diagnosis of upregulated resorption and rapid feedback on efficacy of treatment protocols prior to significant loss of bone in the patient.

Organelles

It has been proven that pH played a pivotal role in many acidic cellular compartments and that the monitoring of pH fluctuations in acidic organelles was essential to investigate the cellular function of the compartments [51]. However, the classic fluorescent dyes (such as fluoresceins, rhodamines and coumarins) were not suitable for studying acidic organelles (such as lysosomes) because their fluorescence is significantly decreased under neutral or acidic conditions. The compound 2-(4-pyridyl)-5-((4-(2-dimethylaminoethyl-aminocarbamoyl) methoxy) phenyl) oxazole (PDMPO) selectively labeled acidic organelles (such as lysosomes) of live cells and the two distinct emission peaks could be used to monitor the pH fluctuations of live cells in ratio measurements. The unique fluorescence properties of PDMPO might give researchers a new tool with which to study acidic organelles of live cells [52].

The combined utilization of three vital fluorescent probes (Hoechst 33342, LysoTracker DND-26, and MitoTracker CMTRos) permitted the dynamic imaging of four organelles during primate spermiogenesis: the nucleus, the mitochondria, the acrosomal vesicle, and the Golgi apparatus [53]. A novel membrane potential sensitive probe (JC-1), with its advantage of its color alteration reversibly from green to red with increasing membrane potentials, could be exploited for imaging live mitochondria on the stage of a microscope [54] .The ratiometric fluorescent pH indicator 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was the most advantageous available probe for the pH of endocytic organelles in live cells [51].

The introduction of LV-pIN-KDEL was anticipated to greatly facilitate a real-time visualization of the structural plasticity and continuous nature of the neuronal endoplasmic reticulum (ER) in healthy and diseased brain tissue [55]. By ER probe erRFP plasmid and caspase-3 probe SCAT3 plasmid, Chen *et al*. [56] found that taxol induced a paraptosis-like programmed cell death in human lung adenocarcinoma (ASTC-a-1) cells by cytoplasm vacuolization due to the swelling of both ER and mitochondria without activating the caspase enzymes. Latex beads are the preferred phagocytic substrate in biochemical studies of phagosome composition and maturation. Caution is needed in extrapolating results from latex bead phagosomes to phagosomes containing physiological substances, especially in early stages of the endocytic pathway [57].

Nucleotides (DNA and RNA)

Imaging products of gene expression in live cells will provide unique insights into the biology of cells. Molecular beacons made attractive probes for imaging mRNA in live cells as they can report the presence of an RNA target by turning on the fluorescence of a quenched fluorophore. There are two methods of linking molecular beacons to tRNA, showing how the joint molecules can be used for imaging an mRNA that is normally present in the cytoplasm in live cultured cells [58]. Multicolor quenched autoligating probes may be of general use in the detection and identification of

sequences in solution, on microarrays, and in microorganisms [59].

As a novel deep red fluorescing bisalkylaminoanthraquinone, DRAQ5 has high affinity for DNA and a high capacity to enter living cells. The fluorescence excitation and emission characteristics of DRAQ5 in living and fixed cells permitted the incorporation of the measurement of cellular DNA content into a variety of multiparameter cytometric analyses [60]. Nucleic acid-templated chemistry is a promising strategy for imaging genetic sequences in living cells. Two new nucleophiles for use in templated nucleophilic displacements with DNA probes were synthesized. The probes were also tested for imaging ribosomal RNA sequences in live *E. Coli* [61].

Chemicals

NO

Nitric oxide (NO) serves as a messenger for cellular signaling. To visualize NO in living cells, a turn-on fluorescent probe combined with microscopy was synthesized. Both the sensitivity to nanomolar concentrations of NO and the spatiotemporal information provided by this complex demonstrate its value for numerous biological applications [62].

Radicals

Radical, especially oxygen free radical, is a major cause of liver injury during reperfusion. BODIPY-alpha-Tocopherol adduct (B-TOH), a novel lipophilic fluorescent antioxidant indicator, is a sensitive and specific probe enabling the molecular imaging of peroxyl radicals in the lipid membranes of live cells [63]. Two-photon confocal microscopy experiments in live macrophages showed that Peroxy Lucifer 1 (PL1), which is a newly synthezied fluorescent probe for imaging hydrogen peroxide produced in living cells by a ratiometric response, can ratiometrically visualize localized hydrogen peroxide bursts generated in living cells at immune response levels [64]. Luminescence analysis has been recently proposed to measure free radical generation by isolated cells or organs, but it allowed only global tissue luminescence. The method using a special saticon videocamera with image intensifier described, allowing the visualization in real time of oxygen free radicals generation on the surface of isolated intact organs, represents a novel and potent tool for the study of oxidative stress [65].

Lipid

Lipids serve as precursors for hormones or other signaling pathways like arachidonic acid derivates, and peptides are able to transfer signals over the membrane either by translocation or by binding to various receptors, which are often integral membrane proteins. The ability to study lipid signaling events has been greatly facilitated by the development of fluorescent molecular imaging techniques. This approach enables investigators to ascertain the involvement of lipid intermediates in diverse signaling pathways [66]. A new probe, which is a cholesterol compound with fluorescent boron dipyrromethene difluoride linked to sterol carbon-24 (BODIPY-cholesterol), closely mimics the membrane partitioning and trafficking of cholesterol and, because of its excellent fluorescent properties, enables the direct monitoring of sterol movement by time-lapse imaging using trace amounts of the probe [67].

Sphingolipid binding domain (SBD) is a small peptide consisting of the SBD of the amyloid precursor protein. It can be conjugated to a fluorophore of choice and exogenously applied to cells, thus allowing for *in vivo* imaging. Fluorescently tagged SBD can be used to investigate the dynamic nature of glycosphingolipid-rich detergent-resistant microdomains that are cholesterol-dependent [68]. The novel probe, named Spy-LHP, reacted rapidly and quantitatively with lipid hydroperoxides to form the corresponding oxide, Spy-LHPOx, which emits extremely strong fluorescence (Phi approximately 1) in the visible range (lambda(em) = 535 nm, 574 nm). These findings establish Spy-LHP as a promising new tool for investigating the physiology of lipid hydroperoxides [69].

The dicyanomethylenedihydrofuran (DCDHF) class of single-molecule fluorophores contains an amine donor and a dicyanomethylenedihydrofuran acceptor linked by a conjugated unit (benzene, naphthalene, or styrene). The photophysical and diffusive behaviors of the DCDHF lipid analogues in membranes were found to be competitive with the well-known lipid probe N-(6-tetramethyl rhodaminethio carbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoet hanolamine [70]. CdTe QDs prepared in water phase would probably become an attractive alternative probe in cellular imaging and bio-labeling [71]. Lipid-enclosed CdSe quantum dots (LEQDs) can function as versatile contrast agents in epi-detection third harmonic generation microscopy for biological applications *in vivo*. Combined with a high penetration 1230 nm laser, these novel features made LEQDs excellent third harmonic generation contrast agents for *in vivo* deep-tissue imaging in the future [72].

Ions

Intracellular zinc levels are homeostatically regulated. Although most is bound, a pool of labile Zn^{2+} is present in cells. The zinc probe FluoZin-3 is useful to monitor zinc fluxes during fluorescent imaging of the trout hepatic cell line D11. With help of this probe, Muylle *et al.* [73] observed the implications for the interpretation of calculated intracellular Zn^{2+} concentrations. Besides, a newly synthesized zinc-sensitive fluorescent probe, N-(6-methoxy-8-quinolyl) -p-carboxybenzoylsulphonamide (TFLZn), was used to monitor intracellular zinc in live rat hippocampal slices. Evidence showed that zinc may play a role in a form of long-term potentiation exhibited by the mossy fibre pathway [74]. Coppersensor-1 (CS1) is a small-molecule, membrane-permeable fluorescent dye for imaging labile copper pools in biological samples, including live cells. This probe, comprising a boron dipyrromethene (BODIPY) chromophore coupled to a thioether-rich receptor, has a picomolar affinity for Cu^{+} ion with high selectivity over competing cellular metal ions [75]. Addtionally, a novel, two-photon probe excited by 880 nm laser photons in response to Mg^{2+} ions, measured the Mg^{2+} ions concentration without interference by Ca^{2+} ions in living cells. The probe was capable of imaging endogenous stores of free Mg^{2+} at a few hundred micrometers depth in live tissues *via* two-photon microscopy [76].

Epithelium Specific Probes

When delivered streptolysin O into living human epithelial cancer cells and primary chicken fibroblasts, multiply

labeled tetravalent RNA imaging probes (MTRIPs) allowed the accurate imaging of native mRNAs and a non-engineered viral RNA, of RNA co-localization with known RNA-binding proteins, and of RNA dynamics and interactions with stress granules [77]. To assess whether immunolabeled nanoparticle biomarkers are comparable to fluorescent marker imaging in measuring epidermal growth factor receptor (EGFR) expression, EGFR was quantified using both imaging methods in cell lines. Immunolabeled nanoparticles quantified receptor expression with performance comparable to fluorescence markers and showed promise to better characterize receptor expression *via* their refractive index sensitivity [78]. According to a study by plasmonic nanoparticles to image EGFR in live cells, dark-field microspectroscopy and labeled plasmonic nanoparticles have the potential utility for LCI [79]. Nanoshell bioconjugates can effectively target and image human epidermal growth factor receptor 2 (HER2), a clinically relevant biomarker, in live human breast carcinoma cells [80].

Cancer Cell Specific Probes

Molecular imaging of live cells provides a unique opportunity to study the growth of tumors. One research revealed that a cyclic peptide, LXY1 had the potential to be developed into an effective imaging and therapeutic targeting agent for human glioblastoma [81]. On the other hand, one major challenge in the evaluation of effectiveness of cancer therapy is the readout for tumor regression and favorable biological response to therapy. *In vivo* NIR imaging of focal cathepsin activity revealed inflammatory reactions etiologically linked with cancer progression and could be a suitable approach for monitoring response to therapy [82].

Gold nanospheres significantly decreased cellular autofluorescence of live cancer cells under physiological conditions when excited at 280nm suggesting targeted gold nanoparticles optical probes could be contrast agents for fluorescence based diagnoses of cancer [83]. High performance *in vivo* magnetic resonance diagnosis of cancer was achievable by improved and multifunctional material properties of iron oxide nanocrystal probes [84]. On top of that, 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl)methoxy)phenyl)oxazole (PDMPO) is another excellent probe for imaging newly deposited silica in living cells and has also a potential for a wide range of applications in various Si-related disciplines, including clinical research (e.g. lung fibrosis and cancer, bone development, artificial bone implantation), and chemistry and physics of materials research [85].

ADVANCE AND WEAKNESS

QDs are useful biological probes because of the increased photostability and quantum efficiency they offer over organic fluorophores. In comparison with fluorescent proteins, QDs have unique optical and elctronic properties, such as size-tunable light emission [86], improved signal brightness [87-90], resistance against photobleaching [91], and simultaneous excitation of multiple fluorescence colors [90].However, toxicity concerns arise since the QD core is composed of cadmium and selenium, both known to be noxious for humans and animals. Knight *et al*. demonstrated species and tissue differences in QD uptake and labeling, and underscore the need for long-term studies of QD toxicity and fate in cells [92].

FUTURE PERSPECTIVES

LCI has great potential value by providing important information of cells *in vivo*. A long term goal in imaging is to monitor the behavior of individual molecules of interest within the living cells of an organism [93]. Various probes and microscopes have been developed in recent years. All these are likely to play a key role in reaching this goal. Researchers still focused on searching adequate probes to lable specific biomolecules. With current interest and concerted efforts of scientists, it is likely that these approaches we discussed here may provide a means to diagnose disease at the earliest and most easily treatable stages, which is expected to improve clinical prognosis.

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REFERENCES

[1] Dufour P, Dufour S, Castonguay A, McCarthy N, De Koninck Y. Two-photon laser scanning fluorescence microscopy for functional cellular imaging: advantages and challenges or one photon is good... but two is better! Med Sci (Paris) 2006; 22: 837-44.

[2] Tokuko H. Live cell imaging: approaches for studying protein dynamics in living cells. Cell Struct Funct 2002; 27: 333-4.

[3] Shaw SL. Imaging the live plant cell. Plant J 2006; 45: 573-98.

[4] Tsien R.Y. The green fluorescent protein. Annu Rev Biochem 1998; 67: 509-44.

[5] Shimomura O. The discovery of aequorin and green fluorescence protein. J Microsc 2005; 217: 3-15.

[6] Amos B. Lessons from the history of light microscopy. Nat Cell Biol 2000; 2: E151-2.

[7] Hell SW. Toward fluorescence nanoscopy. Nat Biotechnol 2003; 21: 1347-55.

[8] Porter G. Some reflections on the work of Theodor Förster. Naturwissenschaften 1976; 63: 207-11.

[9] Thomas MJ, Diane SL, Elizabeth A. Fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM). From cells to proteins: imaging nature across dimensions: Springer: Netherlands 2006.

[10] Mao S, Benninger RK, Yan Y, *et al.* Optical lock-in detection of FRET using synthetic and genetically encoded optical switches. Biophys J 2008; 94: 4515-24.

[11] Treanor B, Lanigan PM, Suhling K, *et al.* Imaging fluorescence lifetime heterogeneity applied to GFP-tagged MHC protein at an immunological synapse. J Microsc 2005; 217: 36-43.

[12] Braet F, Vermijlen D, Bossuyt V, De Zanger R, Wisse E. Early detection of cytotoxic events between hepatic natural killer cells and colon carcinoma cells as probed with the atomic force microscope. Ultramicroscopy 2001; 89: 265-73.

[13] Schaus SS, Henderson ER. Cell viability and probe-cell membrane interactions of XR1 glial cells imaged by atomic force microscopy. Biophys J 1997; 73: 1205-14.

[14] Cappella B, Baschieri P, Frediani C, Miccoli P, Ascoli C. Force-distance curves by AFM. IEEE Eng Med Biol Mag 1997; 16: 58-65.

[15] Mahaffy RE, Shih CK, MacKintosh FC, Käs J. Scanning Probe-Based Frequency-Dependent Microrheology of Polymer Gels and Biological Cells. Phys Rev Lett 2000; 85: 880-3.

[16] Shen Y, Sun JL, Zhang A, Hu J, Xu LX. A new image correction method for live cell atomic force microscopy. Phys Med Biol 2007; 52: 2185-96.

[17] Gorelik J, Zhang Y, Shevchuk AI, *et al.* The use of scanning ion conductance microscopy to image A6 cells. Mol Cell Endocrinol 2004; 217: 101-8.

[18] Zhang Y, Gorelik J, Sanchez D, *et al.* Scanning ion conductance microscopy reveals how a functional renal epithelial monolayer maintains its integrity. Kidney Int 2005; 68: 1071-7.

[19] Novak P, Li C, Shevchuk AI, *et al.* Nanoscale live-cell imaging using hopping probe ion conductance microscopy. Nat Methods 2009; 6: 279-81.

[20] Amos WB, White JG. How the Confocal Laser Scanning Microscope entered Biological Research. Biol Cell 2003; 95: 335-42.

[21] Shevchuk AI, Hobson P, Lab MJ, Klenerman D, Krauzewicz N, Korchev YE. Endocytic pathways: combined scanning ion conductance and surface confocal microscopy study. Pflugers Arch 2008; 456: 227-35.

[22] Diaspro A. Two-photon excitation microscopy. IEEE Eng Med Biol 1999; 18: 16-99.

[23] Pennisi E. Biochemistry: photons add up to better microscopy. Science 1997; 275: 480-1.

[24] Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol 2003; 21: 1369-77.

[25] Girkin JM. Optical physics enables advances in multiphoton imaging. J Phys D: Appl Phys 2003; 36: R250-R8.

[26] Schafer-Hales KJ, Belfield KD, Yao S, Frederiksen PK, Hales JM, Kolattukudy PE. Fluorene-based fluorescent probes with high two-photon action cross-sections for biological multiphoton imaging applications. J Biomed Opt 2005; 10: 051402.

[27] Chip-man Technologies Ltd. Available from: http: //www.chipm-antech.com

[28] Sislab Supply Innovative Solutions for Life Science Research. Available from: http: //nuke.sislab/Home

[29] Melles Griot. Available from: http: //www.mellesgriot.com

[30] Narkilahti S, Rajala K, Pihlajamäki H, Suuronen R, Hovatta O, Skottman H. Monitoring and analysis of dynamic growth of human embryonic stem cells: comparison of automated instrumentation and conventional culturing methods. Biomed Eng Online 2007; 6: 11.

[31] Giepmans BN, Adams SR, Ellisman MH, Tsien RY. The fluorescent toolbox for assessing protein location and function. Science 2006; 312: 217-24.

[32] Tsien RY. Building and breeding molecules to spy on cells and tumors. FEBS Lett 2005; 579: 927-32.

[33] Chen I, Ting AY. Site-specific labeling of proteins with small molecules in live cells. Curr Opin Biotechnol 2005; 16: 35-40.

[34] Alivisators P. The use of nanocrystals in biological detection. Nat Biotechnol 2004; 22: 47-52.

[35] Dabbousi BO, Rodriguez-Viejo J, Mikulec FV, *et al.* (CdSe)ZnS Core−Shell Quantum Dots: Synthesis and Characterization of a Size Series of Highly Luminescent Nanocrystallites. J Phys Chem B 1997; 101: 9463-75.

[36] Pons T, Uyeda HT, Medintz IL, Mattoussi H. Hydrodynamic dimensions, electrophoretic mobility, and stability of hydrophilic quantum dots. J Phys Chem B 2006; 110: 20308-16.

[37] Packard BZ, Artym VV, Komoriya A, Yamada KM. Direct visualization of protease activity on cells migrating in three-dimensions. Matrix Biol 2009; 28: 3-10.

[38] Blum G, Mullins SR, Keren K, *et al*. Dynamic imaging of protease activity with fluorescently quenched activity-based probes. Nat Chem Biol 2005; 1: 203-9.

[39] Suhling K, Siegel J, Phillips D, *et al.* Imaging the environment of green fluorescent protein. Biophys J 2002; 83: 3589-95.

[40] Pick H, Jankevics H, Vogel H. Distribution plasticity of the human estrogen receptor alpha in live cells: distinct imaging of consecutively expressed receptors. J Mol Biol 2007; 374: 1213-23.

[41] Misra P, Lebeche D, Ly H, *et al.* Quantitation of CXCR4 expression in myocardial infarction using 99mTc-labeled SDF-1alpha. J Nucl Med 2008; 49: 963-9.

[42] Bannai H, Lévi S, Schweizer C, Dahan M, Triller A. Imaging the lateral diffusion of membrane molecules with quantum dots. Nat Protoc 2006; 1: 2628-34.

[43] Rajan SS, Liu HY, Vu TQ. Ligand-bound quantum dot probes for studying the molecular scale dynamics of receptor endocytic trafficking in live cells. ACS Nano 2008; 2: 1153-66.

[44] Treiser MD, Liu E, Dubin RA, Sung HJ, Kohn J, Moghe PV. Profiling cell-biomaterial interactions *via* cell-based fluororeporter imaging. Biotechniques 2007; 43: 361-6, 8.

[45] Myers KR, Lo KW, Lye RJ, *et al.* Intermediate chain subunit as a probe for cytoplasmic dynein function: biochemical analyses and live cell imaging in PC12 cells. J Neurosci Res 2007; 85: 2640-7.

[46] Iliev AI, Wouters FS. Application of simple photobleaching microscopy techniques for the determination of the balance between anterograde and retrograde axonal transport. J Neurosci Methods 2007; 161: 39-46.

[47] Gangadhar NM, Stockwell BR. Chemical genetic approaches to probing cell death. Curr Opin Chem Biol 2006; 11: 83-7.

[48] Pham W, Pantazopoulos P, Moore A. Imaging farnesyl protein transferase using a topologically activated probe. J Am Chem Soc 2006; 128: 11736-7.

[49] Maxwell D, Chang Q, Zhang X, Barnett EM, Piwnica-Worms D. An improved cell-penetrating, caspase-activatable, near-infrared fluorescent Peptide for apoptosis imaging. Bioconjug Chem 2009; 20: 702-9.

[50] Kozloff KM, Quinti L, Patntirapong S, *et al.* Non-invasive optical detection of cathepsin K-mediated fluorescence reveals osteoclast activity *in vitro* and *in vivo*. Bone 2009; 44: 190-8.

[51] Overly CC, Lee KD, Berthiaume E, Hollenbeck PJ. Quantitative measurement of intraorganelle pH in the endosomal-lysosomal pathway in neurons by using ratiometric imaging with pyranine. Proc Natl Acad Sci USA 1995; 92: 3156-60.

[52] Diwu Z, Chen CS, Zhang C, Klaubert DH, Haugland RP. A novel acidotropic pH indicator and its potential application in labeling acidic organelles of live cells. Chem Biol 1999; 6: 411-8.

[53] Moreno RD, Ramalho-Santos J, Chan EK, Wessel GM, Schatten G. The Golgi apparatus segregates from the lysosomal/acrosomal vesicle during rhesus spermiogenesis: structural alterations. Dev Biol 2000; 219: 334-49.

[54] Reers M, Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 1991; 30: 4480-6.

[55] Jones VC, McKeown L, Verkhratsky A, Jones OT. LV-pIN-KDEL: a novel lentiviral vector demonstrates the morphology, dynamics and continuity of the endoplasmic reticulum in live neurones. BMC Neurosci 2008; 9: 10.

[56] Chen TS, Wang XP, Sun L, Wang LX, Xing D, Mok M. Taxol induces caspase-independent cytoplasmic vacuolization and cell death through endoplasmic reticulum (ER) swelling in ASTC-a-1 cells. Cancer Lett 2008; 270: 164-72.

[57] Giorgione J, Clarke M. Heterogeneous modes of uptake for latex beads revealed through live cell imaging of phagocytes expressing a probe for phosphatidylinositol-(3,4,5)-trisphosphate and phosphatidylinositol-(3,4)-bisphosphate. Cell Motil Cytoskeleton 2008; 65: 721-33.

[58] Mhlanga MM, Tyagi S. Using tRNA-linked molecular beacons to image cytoplasmic mRNAs in live cells. Nat Protoc 2006; 1: 1392-8.

[59] Sando S, Abe H, Kool ET. Quenched auto-ligating DNAs: multicolor identification of nucleic acids at single nucleotide resolution. J Am Chem Soc 2004; 126: 1081-7.

[60] Smith PJ, Blunt N, Wiltshire M, *et al.* Characteristics of a novel deep red/infrared fluorescent cell-permeant DNA probe, DRAQ5, in intact human cells analyzed by flow cytometry, confocal and multiphoton microscopy. Cytometry 2000; 40: 280-91.

[61] Miller GP, Silverman AP, Kool ET. New, stronger nucleophiles for nucleic acid-templated chemistry: synthesis and application in fluorescence detection of cellular RNA. Bioorg Med Chem 2008; 16: 56-64.

[62] Lim MH, Xu D, Lippard SJ. Visualization of nitric oxide in living cells by a copper-based fluorescent probe. Nat Chem Biol 2006; 2: 375-80.

[63] Khatchadourian A, Krumova K, Boridy S, Ngo AT, Maysinger D, Cosa G. Molecular imaging of lipid peroxyl radicals in living cells with a BODIPY-alpha-tocopherol adduct. Biochemistry 2009; [Epub ahead of print].

[64] Srikun D, Miller EW, Domaille DW, Chang CJ. An ICT-based approach to ratiometric fluorescence imaging of hydrogen peroxide produced in living cells. J Am Chem Soc 2008; 130: 4596-7.

[65] Gasbarrini A, Pasini P, Nardo B, *et al.* Chemiluminescent real time imaging of post-ischemic oxygen free radicals formation in livers isolated from young and old rats. Free Radic Biol Med 1998; 24: 211-6.

[66] Kwok V, Vachon E, Downey GP. Use of fluorescent probes to detect lipid signaling intermediates in macrophages. Methods Mol Biol 2009; 531: 301-28.

[67] Hölttä-Vuori M, Uronen RL, Repakova J, *et al.* BODIPY-cholesterol: a new tool to visualize sterol trafficking in living cells and organisms. Traffic 2008; 9: 1839-49.

[68] Hebbar S, Lee E, Manna M, *et al.* A fluorescent sphingolipid binding domain peptide probe interacts with sphingolipids and cholesterol-dependent raft domains. J Lipid Res 2008; 49: 1077-89.

[69] Soh N, Ariyoshi T, Fukaminato T, Nakajima H, Nakano K, Imato T. Swallow-tailed perylene derivative: a new tool for fluorescent imaging of lipid hydroperoxides. Org Biomol Chem 2007; 5: 3762-8.

[70] Nishimura SY, Lord SJ, Klein LO, *et al.* Diffusion of lipid-like single-molecule fluorophores in the cell membrane. J Phys Chem B 2006; 110: 8151-7.

[71] Weng J, Song X, Li L, *et al.* Highly luminescent CdTe quantum dots prepared in aqueous phase as an alternative fluorescent probe for cell imaging. Talanta 2006; 70: 397-402.

[72] Chang CF, Chen CY, Chang FH, *et al.* Cell tracking and detection of molecular expression in live cells using lipid-enclosed CdSe quantum dots as contrast agents for epi-third harmonic generation microscopy. Opt Express 2008; 16: 9534-48.

[73] Muylle FA, Adriaensen D, De Coen W, Timmermans JP, Blust R. Tracing of labile zinc in live fish hepatocytes using FluoZin-3. Biometals 2006; 19: 437-50.

[74] Budde T MA, White JA, Kay AR. Imaging free zinc in synaptic terminals in live hippocampal slices. Neuroscience 1997; 79: 347-58.

[75] Miller EW, Zeng L, Domaille DW, Chang CJ. Preparation and use of Coppersensor-1, a synthetic fluorophore for live-cell copper imaging. Nat Protoc 2006; 1: 824-7.

[76] Kim HM, Yang PR, Seo MS, *et al.* Magnesium ion selective two-photon fluorescent probe based on a benzo[h]chromene derivative for *in vivo* imaging. J Org Chem 2007; 72: 2088-96.

[77] Santangelo PJ, Lifland AW, Curt P, *et al.* Single molecule-sensitive probes for imaging RNA in live cells. Nat Methods 2009; 6: 347-9.

[78] Crow MJ, Grant G, Provenzale JM, Wax A. Molecular imaging and quantitative measurement of epidermal growth factor receptor expression in live cancer cells using immunolabeled gold nanoparticles. Am J Roentgenol 2009; 192: 1021-8.

[79] Curry AC, Crow M, Wax A. Molecular imaging of epidermal growth factor receptor in live cells with refractive index sensitivity using dark-field microspectroscopy and immunotargeted nanoparticles. J Biomed Opt 2008; 13: 014022.

[80] Loo C, Hirsch L, Lee MH, *et al.* Gold nanoshell bioconjugates for molecular imaging in living cells. Opt Lett 2005; 30: 1012-4.

[81] Xiao W, Yao N, Peng L, Liu R, Lam KS. Near-infrared optical imaging in glioblastoma xenograft with ligand-targeting alpha 3 integrin. Eur J Nucl Med Mol Imaging 2009; 36: 94-103.

[82] Gounaris E, Tung CH, Restaino C, *et al.* Live imaging of cysteine-cathepsin activity reveals dynamics of focal inflammation, angiogenesis, and polyp growth. PLoS ONE 2008; 3: e2916.

[83] El-Sayed I, Huang X, Macheret F, Humstoe JO, Kramer R, El-Sayed M. Effect of plasmonic gold nanoparticles on benign and malignant cellular autofluorescence: a novel probe for fluorescence based detection of cancer. Technol Cancer Res Treat 2007; 6: 403-12.

[84] Huh YM, Jun YW, Song HT, *et al. In vivo* magnetic resonance detection of cancer by using multifunctional magnetic nanocrystals. J Am Chem Soc 2005; 127: 12387-91.

[85] Shimizu K, Del Amo Y, Brzezinski MA, Stucky GD, Morse DE. A novel fluorescent silica tracer for biological silicification studies. Chem Biol 2001; 8: 1051-60.

[86] Alivisators P. Semiconductor clusters, nanocrystals, and quantum dots. Science 1996; 271: 933-7.

[87] Bruchez M, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. Science 1998; 281: 2013-6.

[88] Chan W, Maxwell DJ, Gao XH, Bailey RE, Han MY, Nie SM. Luminescent quantum dots for multiplexed biological detection and imaging. Curr Opin Biotechnol 2002; 13: 40-6.

[89] Chan W, Nie SM. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 1998; 281: 2016-8.

[90] Gao XH, Cui YY, Levenson RM, Chung L, Nie SM. *In vivo* cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 2004; 22: 969-76.

[91] Rhyner MN, Smith AM, Gao X, Mao H, Yang L, Nie S. Quantum dots and targeted nanoparticle probes for *in vivo* tumor imaging. Fundan Biomed Technol 2008; 102: 413-425.

[92] Knight VB, Serrano EE. Tissue and species differences in the application of quantum dots as probes for biomolecular targets in the inner ear and kidney. IEEE Trans Nanobiosci 2006; 5: 251-62.

[93] Patterson GH. Fluorescent proteins for cell biology. Methods Mol Biol 2007; 411: 47-80.

[94] Awais M, Sato M, Umezawa Y. Optical probes to identify the glucocorticoid receptor ligands in living cells. Steroids 2007; 72: 949-54.

[95] Tomasulo M, Yildiz I, Raymo FM. pH-sensitive quantum dots. J Phys Chem B 2006; 110: 3853-5.

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